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13. ABSTRACT *(Maximum 200 words)*

Overexpression of the HER2/*neu* oncogene is found in approximately 30% of breast cancers. The mechanism behind which overexpression of HER2/*neu* promotes cell growth is still unclear. We have found that HER2/*neu* oncogenic signals are able to downregulate the expression levels of $p27^{Kip1}$ and $p57^{Kip2}$ proteins. Immunohistochemistry studies showed that the expression of $p27^{Kip1}$ is remarkably decreased in metastasized breast tumor cells. Moreover, the low expression level of $p27^{Kip1}$ in the HER2/*neu* activated B104-1-1 cells can be reversed by introducing the dominant negative Grb2 construct (ΔN-Grb2). Finally, using pulse-chase analysis and in vitro degradation assays we have shown that HER2/*neu* status or activity indeed affects the protein stability of $p27^{Kip1}$ and $p57^{Kip2}$ via ubiquitination pathway.

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Heng-Yin Yang 5/27/99

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Table of Contents

Introduction

The HER2/*neu* protooncogene (also named as c-erbB2) encodes a growth receptor tyrosine kinase. Amplification/overexpression of HER2/*neu* gene was found in human breast cancer and accounts for 30% of breast cancer (Slamon et al., 1987). Also, HER2/*neu* overexpression correlates with a shorter survival rate in breast cancer patients (Berchuck et al., 1990; Slamon et al., 1987; Slamon et al., 1989). As a protooncogene of receptor tyrosine kinase receptor, HER2/*neu* overexpression may stimulate cell proliferation through tyrosine kinase signaling known to mediate mitogenic signals. Thus, oncogenic signal of HER2/*neu* may stimulate cell cycle progression to cause cancer. Little is known about how HER2/*neu* affect the cell cycle regulators in favor of cell proliferation. On the other hand, p27/p57 encode cyclin-dependent kinases inhibitor that can cause G1 arrest by inhibiting the activities of G1 cyclin-CDK. p27 is a potential candidate of tumor suppressor (Clurman and Porter, 1998). Reduced expression of p27 is frequently detected in human cancers, including breast (Catzavelos et al., 1997; Porter et al., 1997), prostate (Cordon-Cardo et al., 1998), gastric (Mori et al., 1997), lung (Catzavelos et al., 1999), skin (Florenes et al., 1998), and colon cancers (Loda et al., 1997). Decreased p27 protein expression correlates with cancer development and poor survival in breast cancer. However, the exact mechanism behind the reduced p27 incancer remains elusive. The p27 protein is shown to be regulated through ubiquitin-mediated proteasome degradation pathway (Pagano et al., 1995). Therefore, we assume that reduced p27 in breast cancer and other types of cancer may due to enhancement of ubiquitin-mediated p27 degradation. Interestingly, the cancers affected by HER2/*neu* overexpression, including colon, breast, gastric and lung cancers, overlap with cancers with decreased p27 expression, suggesting a correlation between these two molecules. To understand the oncogenic signals of HER2/*neu* in promoting cell proliferation to be involved in tumorigenesis, we assessed the roles of HER2/*neu* signaling pathways in regulating p27/p57 for promoting cell growth and/or transformation. We investigate if the reduced expression of p27/p57 in breast cancer is caused by HER2/*neu* signals involved in deregulating p27/p57 ubiquitination.

Body

Results & Discussion

Task1. To investigate the activity of $p27^{Kip1}$ and $p57^{Kip2}$ in breast tumor cells.

p27 is down regulated in HER2/*neu* overexpressing cells

We found that p27 is expressed at low levels in HER2/*neu* overexpressing cell lines such as MDA-MB-361 and MDA-MB-453 when compared with those do not overexpress HER2/*neu*, such as MDA-MB-435 and MDA-MB-468 (Figure 1). To further confirm this observation by isogenic comparison, we use NIH3T3, B104-1-1 cells (derived from NIH3T3 overexpressing constitutively active HER2/*neu*), MCF7 and HER18 (MCF7 cells overexpressing HER2/*neu*) to examine the role of HER2/*neu* signaling in regulating the protein stability of p27 protein. As demonstrated in Figure 2A, we found that the constitutive active HER2/*neu* causes

the decreased expression of p27 (Figure 2A). p27 expression was rescued by using N-Grb2 (an amino- terminal deletion mutant of Grb2) that can block HER2/*neu* signaling but not by C-Grb2 (a carboxyl-terminal deletion mutant of Grb2) that can not block HER2/*neu* signaling (Xie et al., 1995). HER2/*neu* overexpressing cell line (HER18) also has decreased expression of p27 compared with parental cell line MCF7 (non-HER2/*neu* overexpressing) (Figure 2 B).

HER2/*neu* overexpressing correlates with p27 down-regulation in primary breast cancer samples

We examined paraffin embedded, formalin-fixed breast cancer specimens from at least 50 patients with stage 0-IV disease. Tumor cells that have strong HER2/*neu* staining get less p27 staining (Figure 3A & B). On the other hand, those cells have no or less HER2/*neu* staining contain strong p27 staining (Figure 3C & D). These immunohistochemistry studies have shown that HER2/*neu* overexpression was associated with significant down regulation of p27, and this was statistically significant (Figure 3).

p27/p57 is regulated by ubiquitin-mediated protein degradation pathway

To examine if the low expression levels of p27/p57 in HER2/*neu* overexpressing cells are due to the deregulation of protein degradation pathway at a post-transcriptional step. We obtained several breast cancer cell lines (HER2/*neu* overexpressed vs. non-HER2/*neu* overexpressed) and performed Northern blot analysis. The levels of p27 or p57 transcripts in various cell lines we tested are the same (data not shown). Therefore, downregulation of p27/p57 in the HER2/*neu* overexpressing cell lines is taken place at the post-transcriptional level.

Next, we investigated whether this post-transcriptional regulation is mediated by ubiquitination. Our data clearly demonstrated that both p27 and p57 are ubiquitinated, but C-p57 is resistant to ubiquitination (ubiquitination resulting in the ladder forms in SDS-PAGE) (Figure 4). Poly-ubiquitinated forms of p27 and p57, but not of C-p57, were detected by western blotting in lysates from cells treated with LLnL (N-acetyl-leucinyl-leucinyl-norleucinal, a 26S proteasome inhibitor) for 16 hours. DMSO treatment was used as a control to indicate the result is specific. These results indicated that the region responsible for ubiquitination is located within the first 92 a.a. according to the poly-ubiquitination status of each deletion mutants after treatment with LLnL. Although the pMH201 construct with a deletion at one of the consensus CDK sites was still receiving ubiquitination (Figure 4), this only excludes the involvement of this particular site in regulating ubiquitination via phosphorylation. Our observation that C-p57 is not ubiquitinated suggests that C-p57 is a relative stable protein. Therefore, the N-terminus of p57 may contain a functional domain required for ubiquitination.

The half-life of p27 is decreased in HER2/*neu* overexpressing/activating cells

We used pulse-chase analysis to determine the half-life of p27 protein in NIH3T3, B104-1-1, SW3T3, and SW3T3-X-1 cells (overexpressing HER2/*neu*). As shown in Fig. 5, the half-life of p27 in HER2/*neu* activating B104-1-1 cells was 3.5 fold less than that of NIH3T3 cells, and the status of HER2/*neu* can affect the half-life of p27.

Overexpression and activation of HER2/*neu* clearly decrease the half-life of p27 (Figure 5B and C).

HER2/*neu* overexpressing/activation increases the turnover of p27/p57 through ubiquitination

To examine if HER2/*neu* activate the proteolysis system to regulate protein stability of p27 and p57, we prepared cell lysates from B104-1-1 and NIH3T3 cells for an in vitro degradation assay. The cell lysates prepared from B104-1-1 cells can degrade affinity purified Flag-tagged p27 and p57 proteins much more quickly than the lysates from NIH3T3 cells (Figure 6A & B), suggesting that HER-2 activity is important in regulating the protein stability of p27/p57. Interestingly, the C-p27 truncated protein is very stable in either NIH3T3 or B104-1-1 lysates (Figure 6A). These results are consistent with the observation that HER2/*neu* overexpressing cells have low expression of p27/p57 and the N-terminal domain of p27 or p57 is required for ubiquitination process.

We have found that HER2/*neu* oncogenic signals indeed affect the protein stability of p27/p57 proteins via ubiquitination. It is a great strategy that cancer cells have a strong activity toward degradation of negative cell cycle regulators such as p27 and p57 in order to promote cell proliferation. Here we have shown that HER2/*neu* overexpressing breast cancer cells or cell lines have decreased expression levels of p27. It is important to find out how HER2/neu signals up-regulate ubiquitin-mediated protein degradation pathway and what the specific ubiquitin ligases responsible for p27 and p57 protein degradation. It is known that MAP kinase is at the downstream of the HER2/*neu* signaling pathway. It may be involved in phosphorylation of either p27 or p57 protein for degradation. It has been shown that cdk2 is able to phosphorylate p27 and this phosphorylated form of p27 is less stable than non-phosphorylated form. It will be very interesting to know that if the dominant negative cdk2 can block ubiquitination of p27, and therefore increasing the p27 protein level in both in vivo ubiquitination and in vitro degradation assays. We do not yet know whether the phosphorylation of p57 by cdk2 is involved in p57 protein degradation.

Our studies also demonstrated that the N-terminal truncated p27 and p57 (C-p27 and C-p57) are not ubiquitinated, and they are much more stable than the wild type ones. These findings indicate that the N-terminal part of p27 or p57 is necessary for ubiquitination. Our previous study has shown that both C-p27 and C-p57 are not able to inhibit cdk2 activity since the inhibitory domain is deleted. On the other hand, both C-p27 and C-p57 can increase cdk2 kinase activity. This implies that in cancer cells there maybe a mechanism to create an N-terminal truncated p27 and p57 (C-p27 and C-p57), and these truncated forms of p27/p57 can facilitate cell proliferation through their stability (longer half-life) and ability to enhance cdk2 activity.

Task 2. **To investigate effect of p27 and p57 on growth and tumorigenicity of breast cancer cells.**

Recombinant p27/p57 adenovirus construction

We used adenoviral vector as gene transfer system. Adenoviral vectors are attractive vectors for the delivery of foreign genes to mammalian cells for gene therapy. We used an efficient and flexible adenoviral system with deletions in early regions 1 and 3 (Bramson et al., 1996). In order to create p27 and p57 adenoviral expression plasmids, a plasmid that overexpresses p27 or p57 under CMV promoter control was used for cloning. The strategy is to clone the p27 or p57 cDNA to the pCSA vector with CMV promoter first, and then proper restriction sites on the pCSA vector were used for cloning p27 or p57 into the adenoviral vector, pΔE1Sp1A (Microbix). The pΔE1Sp1A-p27 and pΔE1Sp1A-p57 were generated by two steps. First, the CMV5-p27/-p57 plasmids were digested with BamHI/SalI and blunt-ended with Klenow. The fragment contains either p27 or p57 were blunt-end cloned into pCSA (CSA-p27/-p57). Second, The CMV-p27/-p57 fragments were isolated from the CSA-p27/-p57 plasmids using BamHI/XhoI digestion, and these fragments were ligated with the adenoviral vector, pΔE1Sp1A digested with BamHI/SalI. We have already cloned the human c-DNA of p57 and p27 into adenoviral pΔE1Sp1A vectors and are in the process of producing recombinant viruses.

Key Research Accomplishments

- **p27 is downregulated in HER2/*neu* overexpressing cells.**
- **HER2/*neu* overexpressing correlates with p27 down regulation in primary breast cancer samples.**
- **The half-life of p27 is decreased in HER2/*neu* overexpressing/activating cells.**
- **HER2/*neu* overexpressing/activation increases the turnover of p27/p57 through ubiquitination.**
- **Construct recombinant adenovirus of p27/p57**

Reportable outcomes

Meeting Abstract:

1. Oncogenic signals in regulating cell cycle inhibitors. American Association for Cancer Research, 1998

2. HER2/*neu* oncogenic signals in regulating expression of the KIP proteins. Trainee Recognition Day at the University of Texas, M. D. Anderson Cancer Center, Houston, TX, 1999.

Conclusions

These studies elucidate the mechanism of HER2/*neu* oncogenic signals in modulating functions of p27 and p57 through abnormal regulation of ubiquitination. It is anticipated that our studies will shed light on the involvement of p27/p57 in HER2/neu overexpressing breast cancer and may provide potential novel therapeutic strategies to cure breast cancer.

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Appendices

Figures:

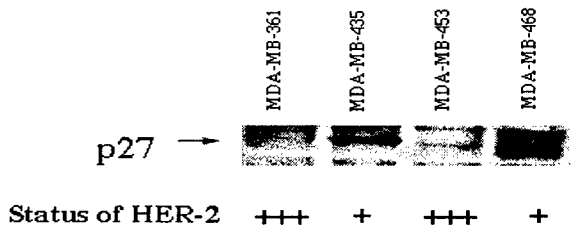


Figure 1. Low-level expression of p27 in HER-2/*neu* overexpressing human breast cancer cell lines. Equal amounts of indicated breast cancer cell lysates were separated by SDS-PAGE and then immunoblotted with p27 antibody. HER-2/*neu* protein status from each breast cancer cell lines is shown as +++ (overexpressing) or + (non-overexpressing).

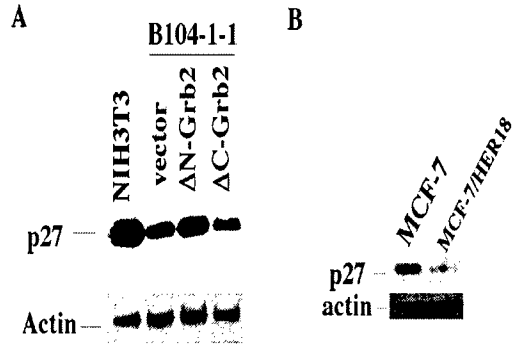


Figure 2. Expression of p27 in HER2/*neu* activating or HER2/*neu* non-activating cell lines. Equal amounts of indicated cell lysates were separated by SDS-PAGE and then immunoblotted with p27 antibody. HER2/*neu* in B104-1-1 cells is constitutively active. ΔN-Grb2, ΔC-Grb2, and vector control are stable transfectants of B104-1-1 cells. HER18 cells derived from MCF7 overexpressing the HER2.

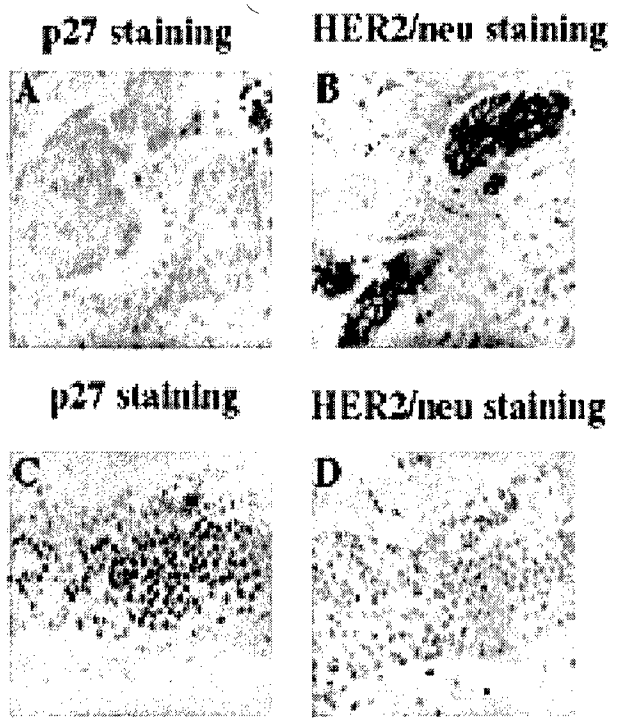


Figure 3. Expression of HER2/*neu* and p27 in breast adenocarcinomas. Immunohistochemistry studies of primary breast adenocarcinomas using anti-p27 (Panels A, C) and anti HER2/*neu* antibodies (Panels B, D). The sections shown in Panels A and B were obtained from the same patient; both overexpressed HER-2/*neu*. The sections shown in Panels C & D were obtained from another patient; neither overexpressed HER-2/*neu*.

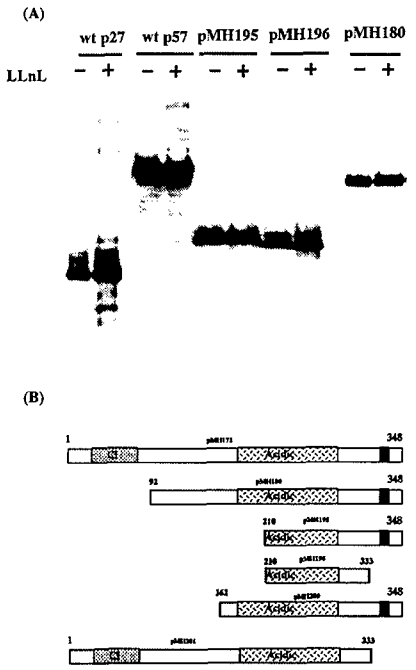


Figure 4. Ubiquitination of p27 and p57.
A, Ubiquitination of p27 and different constructs of p57. R1B/L17 cells were transfected with a vector containing p27, p57, or indicated deletion constructs of p57. After transfection for 24 hours, cells were treated or untreated with LLnL (proteasome inhibitor) for 16 hours. Cell lysates were separated on SDS-PAGE and immunoblotted with p27 or p57 antibody. Ladder forms of protein are poly-ubiquitinated. DMSO treatment was used as a untreated control.
B, Deletion mutants of p57. Different deletion constructs are fused in frame to the Flag epitope in the bacterial overexpression plasmid pET11d (Novagen) or the mammalian expression vector pCMV5. Names of each constructs are denoted.

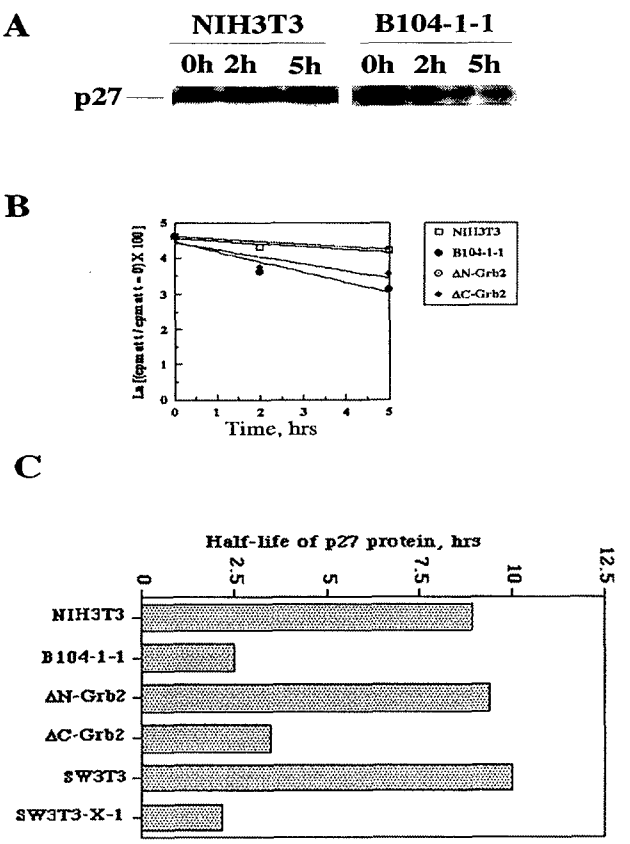
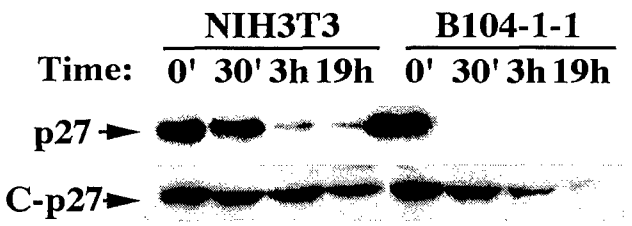


Figure 5. Determination of the half-life of p27 in different cell lines.
A, Pulse-chase analysis of p27 protein in NIH3T3 and B104-1-1 cells. Cells were pulse labeled with 35S Met for 3 hours and chased with cold Met for the indicated hours. Radiolabeled p27 was immunoprecipitated by using p27 antibodies.
B, Effect of HER2/neu activity on stability of the p27 protein. Pulse-chase labeling procedure was used to analyze the protein stability for the p27 protein in the indicated cell lines. Images of the p27 protein were analyzed and quantitated using phosphoimager (Molecular Dynamics). The ratio of labeled p27 protein at a given time point over the labeled p27 protein at time zero was plotted as a function of time. The slope of the resulting curve is used to calculate the half-life of the p27 protein according to procedure previously described (Yang and Evans, 1995). T1/2 = ln2/slope (0.693/K).
C, Half-life of p27 in indicated different cell lines with different HER2/neu status. The half-life of p27 was calculated using the methods described in B.

(A)



(B)

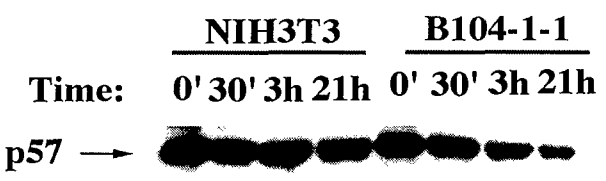


Figure 6. In vitro degradation of p27, Cp27, and p57.
100 ug of cell lysate prepared from NIH3T3 or B104-1-1 cells was incubated with 100 ng of affinity purified Flag-tagged p27 or p57 protein at 37°C for 0', 30', 3 hrs, 19 hrs in a buffer containing Tris, MgCl2, ubiquitin, ATP regenerating system. The level of p27, C-p27, or p57 in each reaction was detected with anti-M2 in a Western Blot.

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